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DETERMINATION OF NUCLEOTIDES BY LIQUID CHROMATOGRAPY WITH A PHO--ETC(U)
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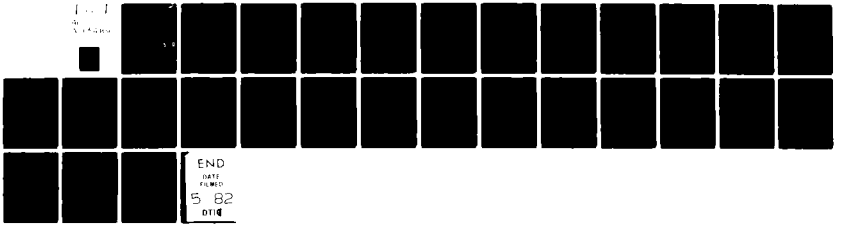

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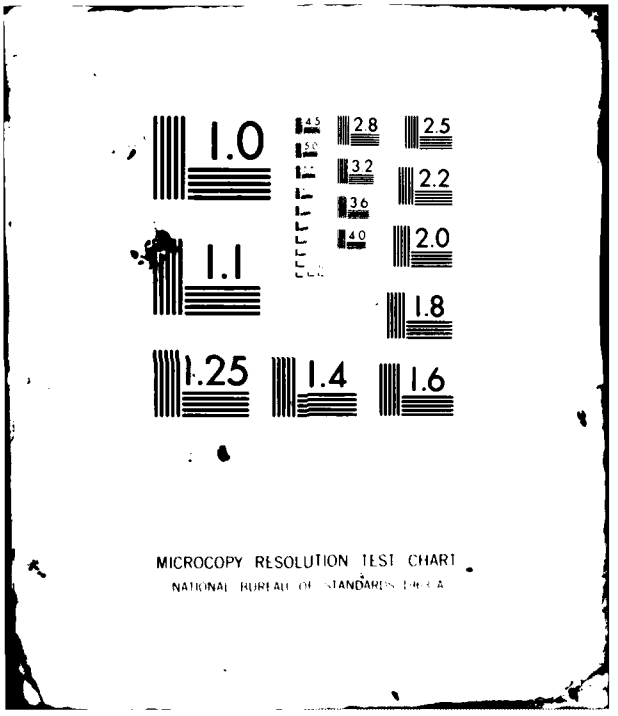
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Nucleotide samples (100 µL) are separated on an anion exchange column using acetate buffers. Selective detection is achieved by observing P(I) emissions. The detection limit for phosphorus is 750 ng.

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Contract N00014-75-C-0513

Task No. NR 051-549

TECHNICAL REPORT NO. 26

DETERMINATION OF NUCLEOTIDES BY LIQUID CHROMATOGRAPHY WITH A PHOSPHORUS SENSITIVE INDUCTIVELY COUPLED PLASMA DETECTOR

by

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Prepared for Publication
in
Analytical Chemistry

DETERMINATION OF NUCLEOTIDES BY LIQUID CHROMATOGRAPHY WITH A PHOSPHORUS SENSITIVE INDUCTIVELY COUPLED PLASMA DETECTOR

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Brief

Nucleotide samples (100 µL) are separated on an anion exchange column using acetate buffers. Selective detection is achieved by observing P(I) emissions. The detection limit for phosphorus is 750 ng.

ABSTRACT

Quantitative analysis of 100 µL nucleotide samples is accurately performed by analyzing total phosphorus concentration using an inductively coupled plasma (ICP) system. A single calibration using Na_2HPO_4 (aq) is useful for all nucleotides. A detection limit of 750 ng of phosphorus is determined with an RSD of 4.5%. High performance liquid chromatography (HPLC) is used to separate mixtures of nucleotides on an anion exchange column using acetate buffers. The ICP is used as a selective detector by observing P(I) emissions at 213.6 nm. A Babington nebulizer which is used to aspirate the high percentage salt solutions with 6% efficiency is described.

INTRODUCTION

Nucleotides, the monomeric units which compose important macromolecules such as DNA and RNA, can be efficiently separated by high performance liquid chromatography (HPLC) using anion exchange columns due to the presence of one or more terminal phosphate groups in each molecule (1, 2). Quantitative determinations normally involve absorbance measurements at 254 nm and comparison to a carefully determined calibration curve. Since each type of nucleotide has a unique extinction coefficient, it is necessary to calibrate the instrument for each compound individually. This involves preparation and measurement of a series of standards for each nucleotide of interest. This procedure is aggravated by the hygroscopic and unstable nature of nucleotides which must usually be dessicated and frozen until used.

The use of a detector which requires only a single calibration, ideally with a primary standard, for the entire group of nucleotides would clearly be advantageous. Also, a detector which is selective for phosphorus would provide additional assurance that similar compounds such as nucleosides would not result in erroneous data.

Elemental analysis has been shown to be a viable alternative for the quantitative analysis of organic coumpounds using an inductively coupled plasma (ICP) (3, 4, 5). Several properties of the ICP make it well-suited for this type of application: high sensitivity, large dynamic range, few chemical interferences, and multi-element capability (6,7).

Windsor and Denton have recently demonstrated that elemental analysis using an ICP is a practical detector for gas chromatography (8, 9). The use of argon as a carrier gas permitted complete empirical formula

determinations of a variety of hydrocarbons and metallo-organic compounds. The analysis of nitrogen and oxygen in compounds separated by GC has also been reported (10, 11).

Since it is not practical to determine those elements in analyte species which are also present in the mobile phase, these same capabilities using the ICP as an HPLC detector have not been realized. However, the analysis of elements in compounds which are not present in the liquid mobile phase has been reported using the ICP as a liquid chromatography detector (12, 13, 14). Quantitative results were presented for a number of elements including iron, mercury, lead and phosphorus.

This paper presents the results of studies undertaken to assess the capabilities of an ICP used as a phosphorus sensitive HPLC detector for the quantitative analysis of nuclectides. Operational parameters such as nebulizer design and use of acetate mobile phases are discussed. General characteristics including sensitivity, the effects of flow rate and selectivity are investigated.

EXPERIMENTAL SECTION

The instrumentation used in this work is described in Table I. The column effluent was directed through the UV absorption detector prior to entering the ICP. This allowed direct comparisons of the response of each detector, which was helpful in analyzing the system performance. The analyte solution was aerosolized for introduction into the ICP with a Babington principle nebulizer (17, 18, 19), modified as shown in Figure 1. A 0.2mm exit orifice in the Kel-F sample gas

inlet produces a high velocity gas jet at the point the solution enters. The resulting aerosol is impinged upon a glass impactor for droplet refinement. A relatively small (20 mL) glass spray chamber was used to minimize peak broadening. The clog-free nature of this type of nebulizer makes it well-suited for applications involving high salt samples were long term stability is required. Solutions containing 30% NaCl (saturated) have been nebulized for long periods of time without clogging. A nebulization efficiency of 6% (the difference between the amount of solution entering and the amount collected in the drain) was measured for aqueous samples at a flow rate of 1.5 mL/min with a 0.7 L/min (20 psig) gas flow rate. The efficiency did not appear to change after extended periods of use unlike the nebulizer previously reported by Gustavsson (20).

The mobile phases used in this work were prepared in distilled deionized water using research grade sodium acetate (Lehigh Valley Chemical Co., Easton, PA). The pH was adjusted with glacial acetic acid (VWR Scientific, San Francisco, CA). Immediately prior to use, each solution was filtered through a Millipore, Corp. (Bedford, MA) HAWP 047-00 0.45 um filter. No degassing was found to be necessary.

AR grade disodium phosphate (Mallinckrodt Chemical Works, St. Louis, MO) was used without further purification for preparation of phosphorus standards. Weighted samples were taken up in distilled deionized water.

Nucleotide samples obtained from Sigma Chemicals (St. Louis, MO) were dissolved in 0.01M tris(hydroxy methyl)aminomethane THAM after weighing. These solutions were refrigerated when not being used.

RESULTS AND DISCUSSION

The optimum observation position in the discharge was determined while aspirating a solution of 100 ppm phosphorus in 0.3M sodium acetate. The net phosphorus signal (gross signal at 213.6 nm minus background at 213.8 nm) was recorded at equally spaced intervals along the vertical axis of the torch with the aid of the microcomputer. The optimum viewing height was chosen at the position of maximum signal-to-background ratio (SBR). The same procedure was used to locate the optimum horizontal viewing position. Other parameters such as r. f. power, sample gas flow rate, and photomultiplier tube voltage were held constant.

In order to evaluate the transient performance of the ICP under controlled chromatographic conditions the anion exchange column was replaced with an equal length of 316 stainless steel tubing with an internal diameter of 0.23 mm. Injections made onto this "pseudo column" are therefore unretained and give rise to peaks with a FWHM of approximately 15 s, depending upon the flow rate.

Seven standards ranging from 10 - 1000 ppm were used to determine the calibration curve. The emission signal at 213.6 nm resulting from each 100 µL injection was integrated using CONVERS(21) software and plotted against the weight of phosphorus in the respective sample. The emission signal was found to be independent of the mobile phase employed (i.e. A or B in Table I). A correlation coefficient of 0.99 was calculated using linear regression analysis on the data obtained in this manner. A relative standard deviation of 4.5% was measured by integrating the emission signal obtained from a series of ten injections

of 10 uG of phosphorus. A detection limit of 750 nG of phosphorus was calculated according to the method of Skogerboe and Grant, with the criteria of a signal equal to twice the standard deviation of the measurement(22).

The quantitative results of phosphorus analysis for several nucleotides are listed in Table II. The calculated phosphorus content is based on the amount of the respective compound used, its molecular weight and the purity as given by the manufacturer. The samples were weighed and dissolved in THAM buffer upon arrival to prevent degradation and/or water absorption. The samples were assumed to be anhydrous for these calculations. The observed phosphorus content was obtained by integrating the emission signal from 100 µL injections of each nucleotide solution and comparison to the analytical curve as shown in Figure 2(b). In most cases there is good agreement, within the standard deviation. The relatively large difference shown by cytidine monophosphate (CMP) may be due to water absorption prior to weighing, although this was not verified.

The results in Table II demonstrate that it is possible to quantitatively analyze nucleotides in an ICP using a single calibration based on a primary standard such as Na_2HPO_4.

The integrated emission signal measured on 10 µG phosphorus was found to depend upon solution flow rate as shown in Figure 3. This characteristic, due primarily to a change in nebulizer efficiency at different solution flow rates is common to ICP nebulizers.

It is desirable from a chromatography standpoint to use higher flow rates while the ICP operates more efficiently at lower flow rates.

Therefore, a compromise is necessary in this type of application.

An anion exchange column was used to separate mixtures of nucleotides. The strong sodium acetate buffer listed as solvent B in Table I, was pumped through the column to remove all phosphorus prior to use with the nucleotides. A 100 µL mixture containing 6 µg phosphorus as CMP and 9 uG as UMP (uridine monophosphate) was injected onto the column. A ten minute linear gradient from the 80 to 100% solvent B, and a 1.0 mL/min flow rate resulted in the chromatogram shown in figure 4. The two analyte peaks are found to be sharp and symmetrical indicating the stability of the nebulizer system in this application.

A comparison of the UV and ICP detectors is shown in figure 5, which was obtained by separation of a three component mixture using a five minute linear gradient and a flowrate of 1.5 mL/min. The generally non-specific nature of the UV detector (lower tracing) is demonstrated by the presence of both a solvent peak, a, and a peak due to adenosine, b, a nucleoside which does not contain a phosphate group. These two peaks are not present in the ICP recording (upper trace) because neither of these compounds contains phosphorus. Both detectors respond to the nucleotides AMP (adenosine monophosphate), CMP and UMP, respectively, which correspond to peaks c, d and e. The slight time lag between the UV and ICP recordings is due to the physical separation of the two detectors as previously described.

Inorganic phosphate, presumably the result of partial hydrolysis of one or more nucleotides, is responsible for peak f in the ICP output demonstrating the advantage of two detectors.

The di- and triphosphate adenosine nucleotides (ADP and ATP) had much longer retention times than the monophosphate nucleotides; ADP required 35-40 minutes while ATP was not removed from the column even after an hour. Guanosine monophosphate (GMP) was successfully analyzed in this system but could only be partially separated from CMP under the conditions employed.

The detection limits and precision were somewhat degraded following separation on the columns. Although the sensitivity of the ICP system for nucleotides (based on phosphorus results) is less than that of the UV detector, it can provide valuable additional information as an LC detector. Applications involving compounds other than nuclectides may also benefit from the information provided by an ICP system.

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ACKNOWLEDGMENTS

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TABLE I

INSTRUMENTATION AND EXPERIMENTAL PARAMETERS

COMPONENT	MODEL, MANUFACTURER	PARAMETER
HPLC	Model 5000 Liquid Chromatograph, Varian Instruments, Walnut Creek, CA (100 uL sampling Loop, 254 nm absorption detector).	
Column	MicroPak AX-10 anion exchange column, Varian Instruments, Walnut Creek, CA (30 cm).	
Solvents	A: 0.007M $NaC_2H_3O_2$, pH 4.00 B: 0.3M $NaC_2H_3O_2$ / 0.3M NaCl, pH 3.00	5 min linear gradient from A to B.
ICP torch assembly and rf generator	Laboratory constructed, demountable torch assembly (15). 204C-1 Linear Power Amplifier, Collins Radio, Cedar Rapids, IA (16).	1.0 - 3.0 kW 15 L/min Ar coolant, 0.5 L/min Ar auxiliary.
Nebulizer	Laboratory constructed (Figure 1). See text.	30 psi, 1.0 L/min.
Monochromator	EU-700 Scanning Monochromator, GCA-McPherson, Acton, MA. (0.35 M, f6.8, 250 nm blaze, 2 nm/mm, modified with stepper motors for computer control).	213.6 nm 85 um slit width; 3 mm height.
Photomultiplier Tube (PMT)	R166UH Solar Blind, Hamamatsu, Middlesex, NJ.	
PMT Voltage Source	Model HV-1565 High Voltage Regulated Power Supply, Pacific Designs Instrument Co., San Jose, CA.	-1000 vdc

TABLE 1 (Continued)

COMPONENT	MODEL, MANUFACTURER	PARAMETER
Current-Voltage Converter	Laboratory constructed using Signetics Corp NE536 Operational Amplifier, Sunnyvale, CA.	1 V/uA 0.1 s RC
Recorder	Model 255 Strip Chart Recorder, Linear Instruments, Irvine, CA.	1 Vfs 1 cm/min
Computer	Altair 8800 Microcomputer, MITS, Albuquerque, NM. (Intel 8080 Microprocessor, 16K RAM). (North Star minifloppy, CONVERS 2.1 operating system).	

TABLE II DETERMINATION OF NUCLEOTIDES WITH AN ICP

Nucleotide	Calculated Phosphorus Content[a] µG	Observed Phosphorus Content uG	Recovery %
AMP	18.1	18.7	103
ADP	32.9	30.7	93
CMP	10.9	9.3	85
UMP	18.5	17.5	95
ATP	35.7	36.9	103
GMP	15.1	14.1	93

[a] Calculated from weight of compound and purity.

FIGURE CAPTIONS

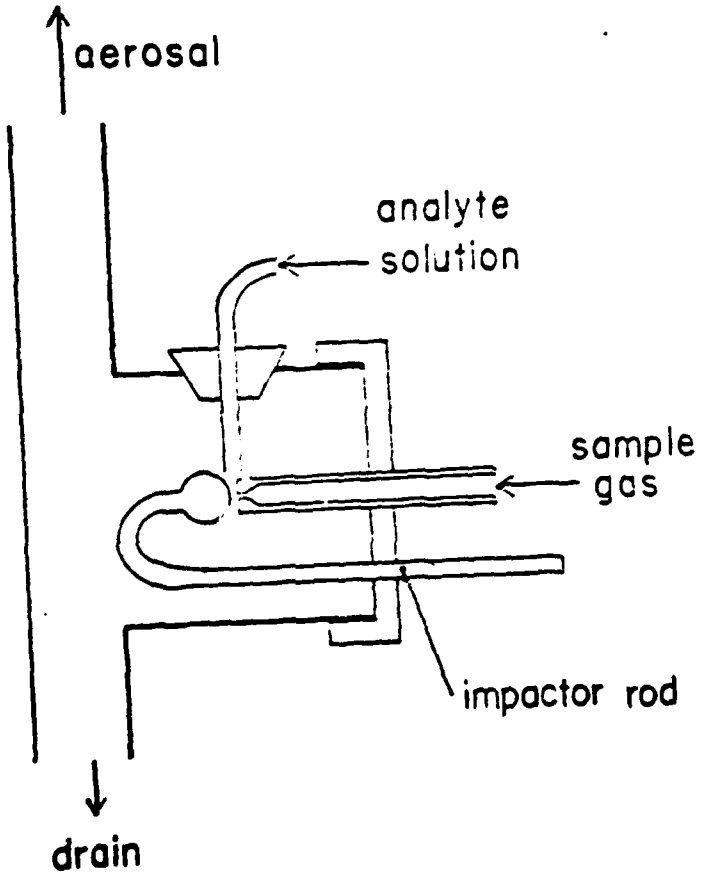
Figure 1. Babington principle nebulizer optimized for use with the HPLC-ICP system.

Figure 2. Calibration curve obtained by integrating the 213.6 nm P(I) emission signal resulting from 100 µL injections of phosphorus standards with nucleotide results

Figure 3. The flow rate cependence of the integrated P(I) emission signal resulting from 100 µL injections of 10 µg of phosphorus.

Figure 4. Separation of two nucleotides by anion exchange chromatography using the ICP as a phosphorus sensitive detector.

Figure 5. Comparison of a UV and an ICP detector for the separation of three nucleotides using a 5 min linear gradient elution. Solvent peak (a), nucleoside (b), AMP (c), CMP (d), UMP (e), and $H_2PO_4^-$ (f).



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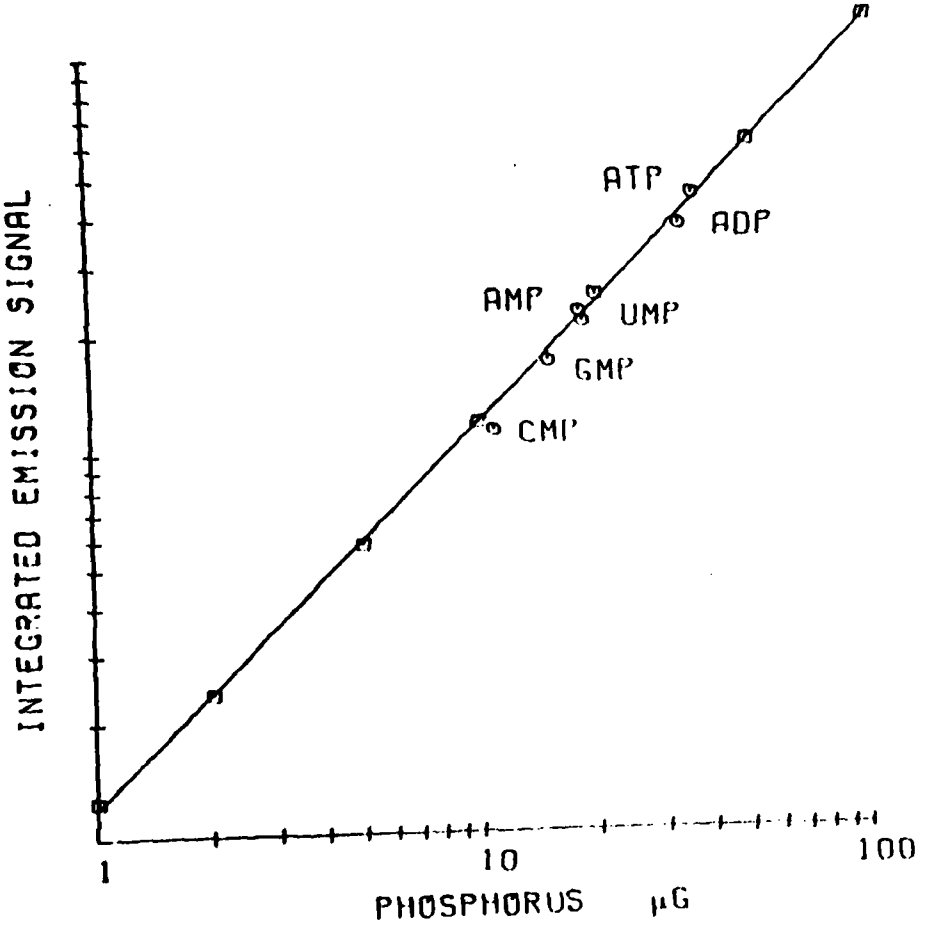
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PHOSPHORUS µG

Figure 2

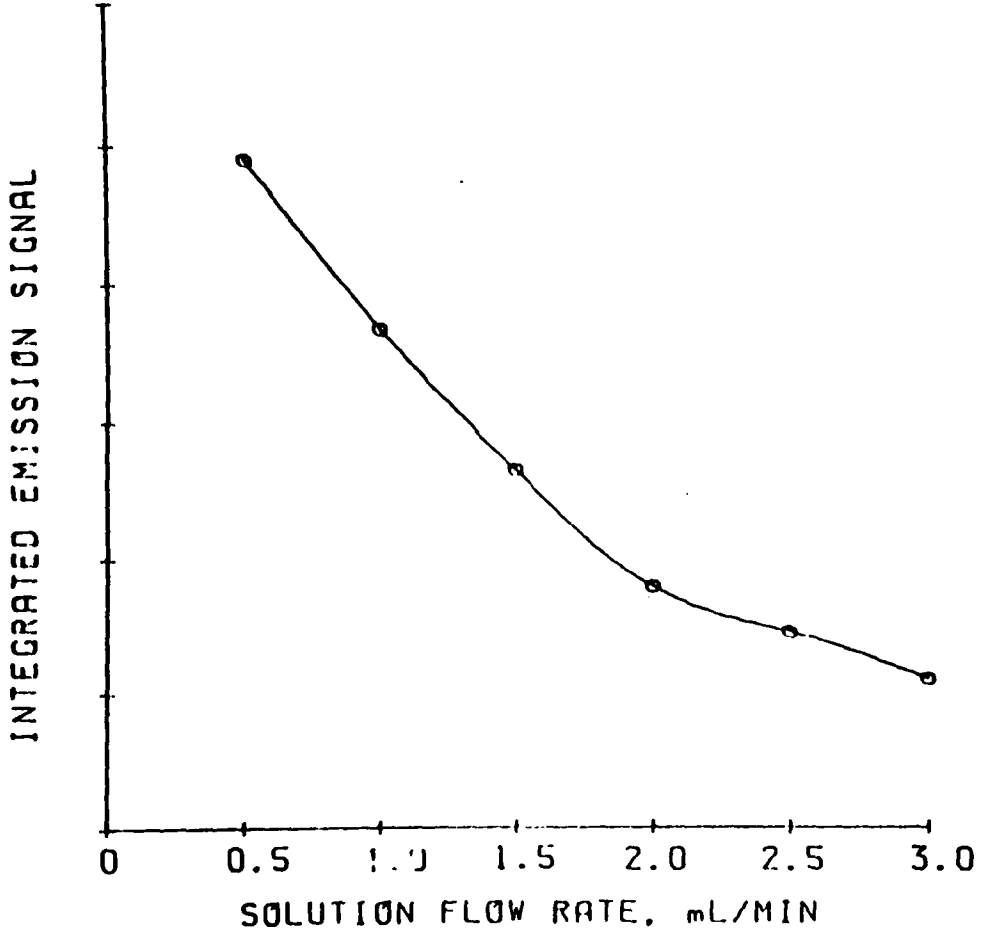
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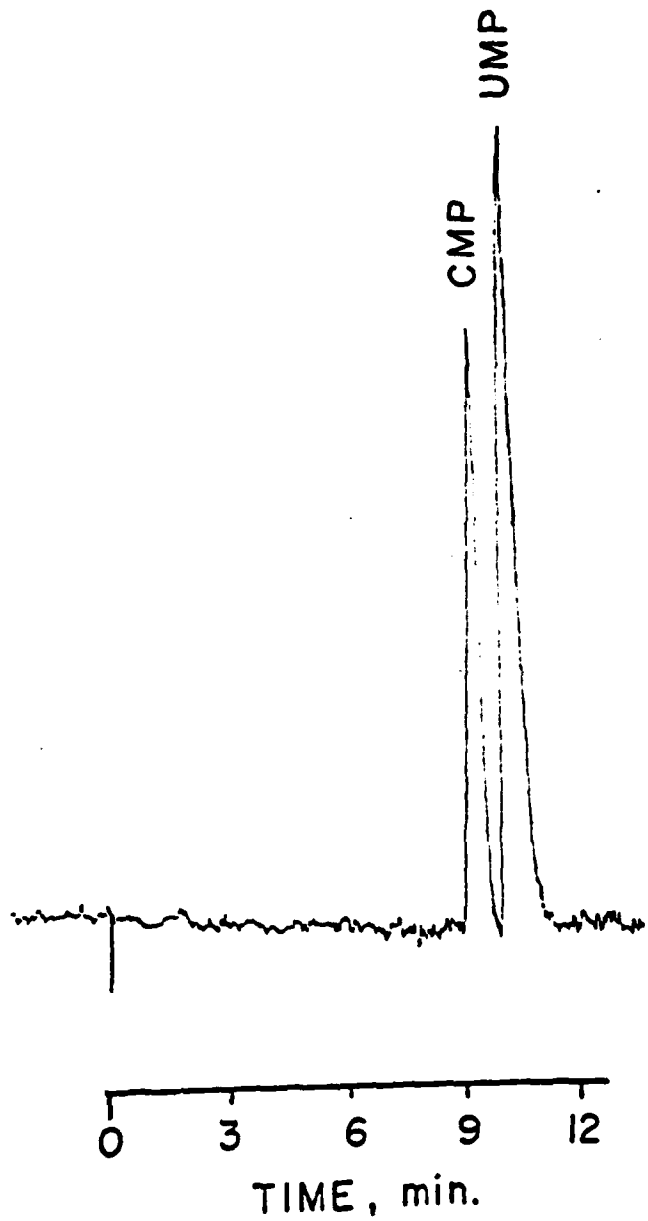
Figure 3



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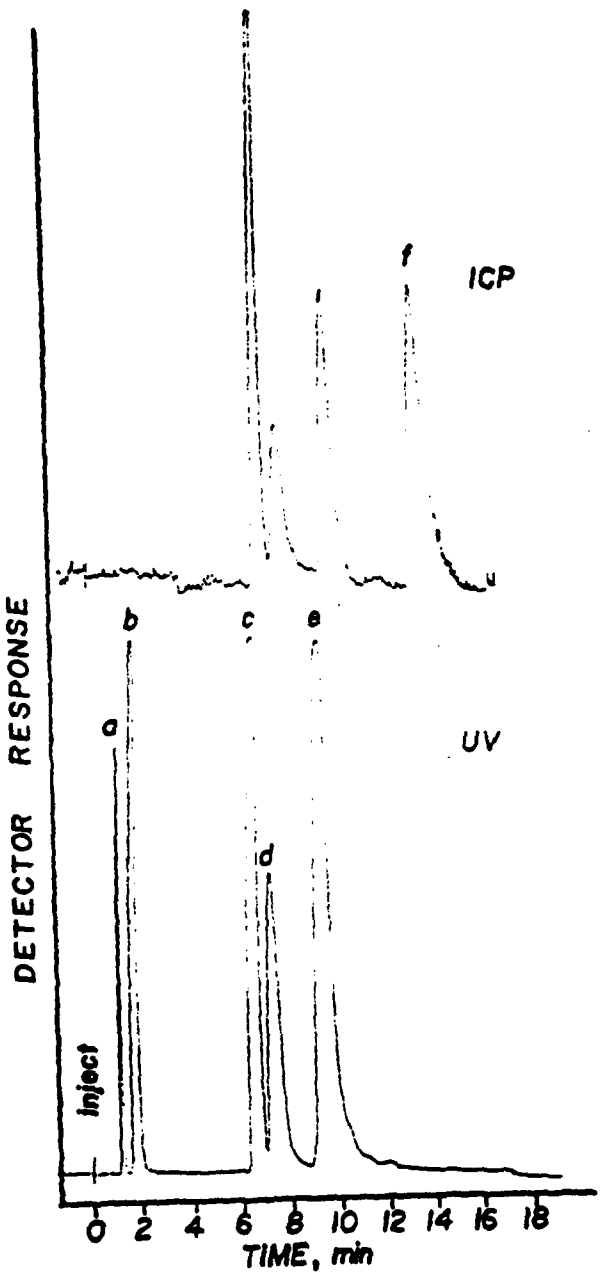
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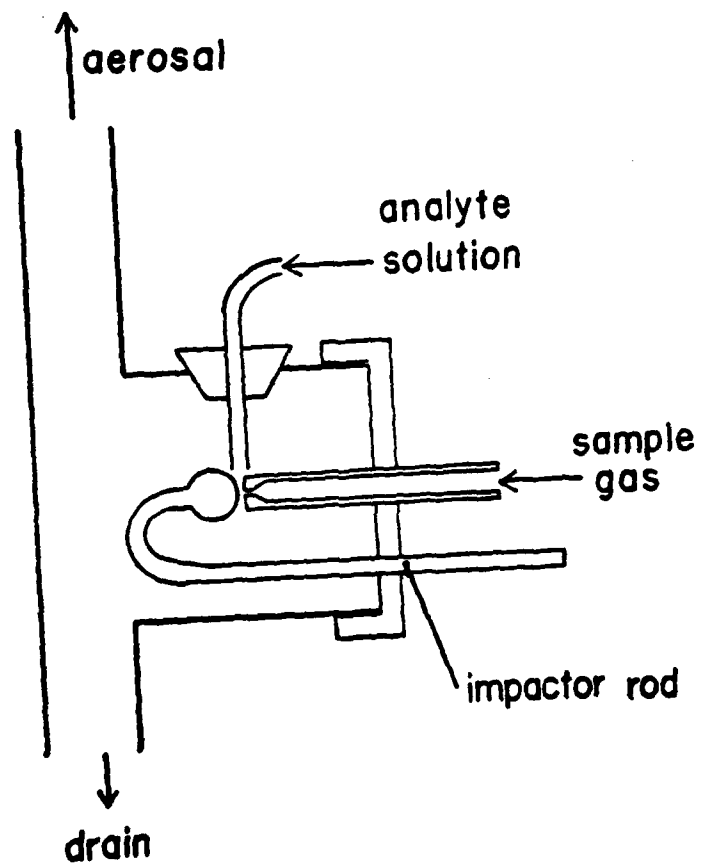
Figure 5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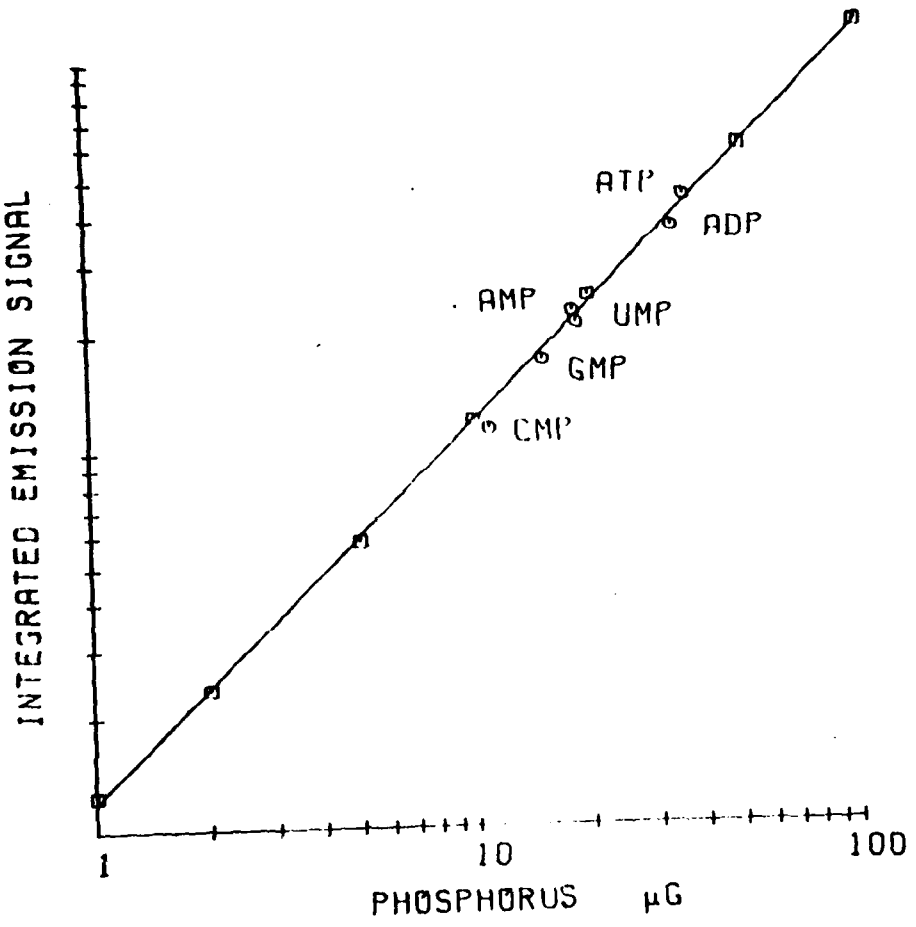
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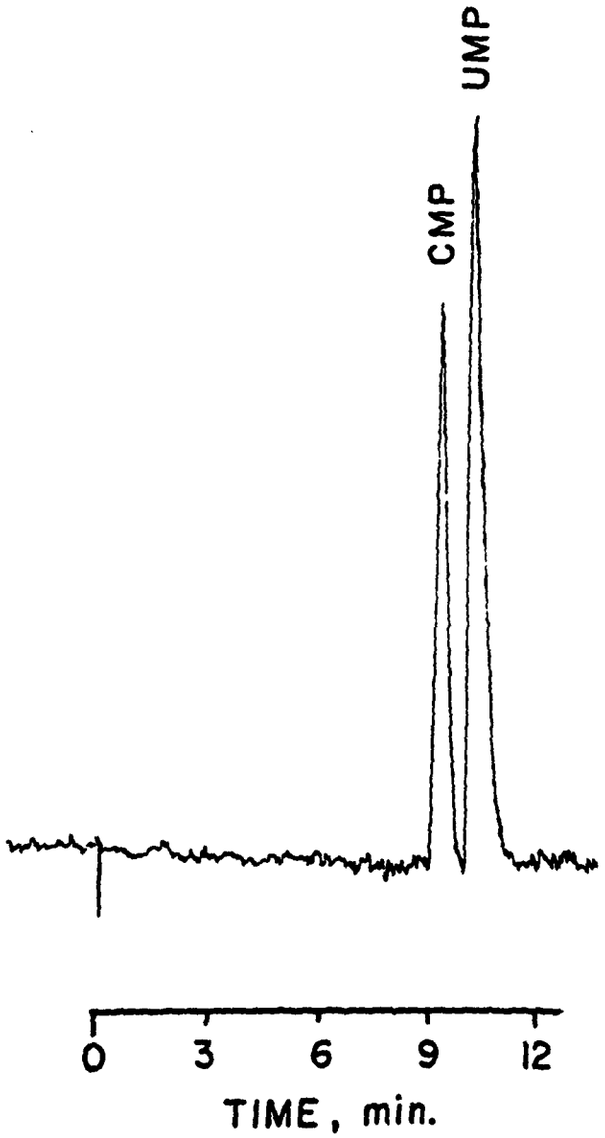
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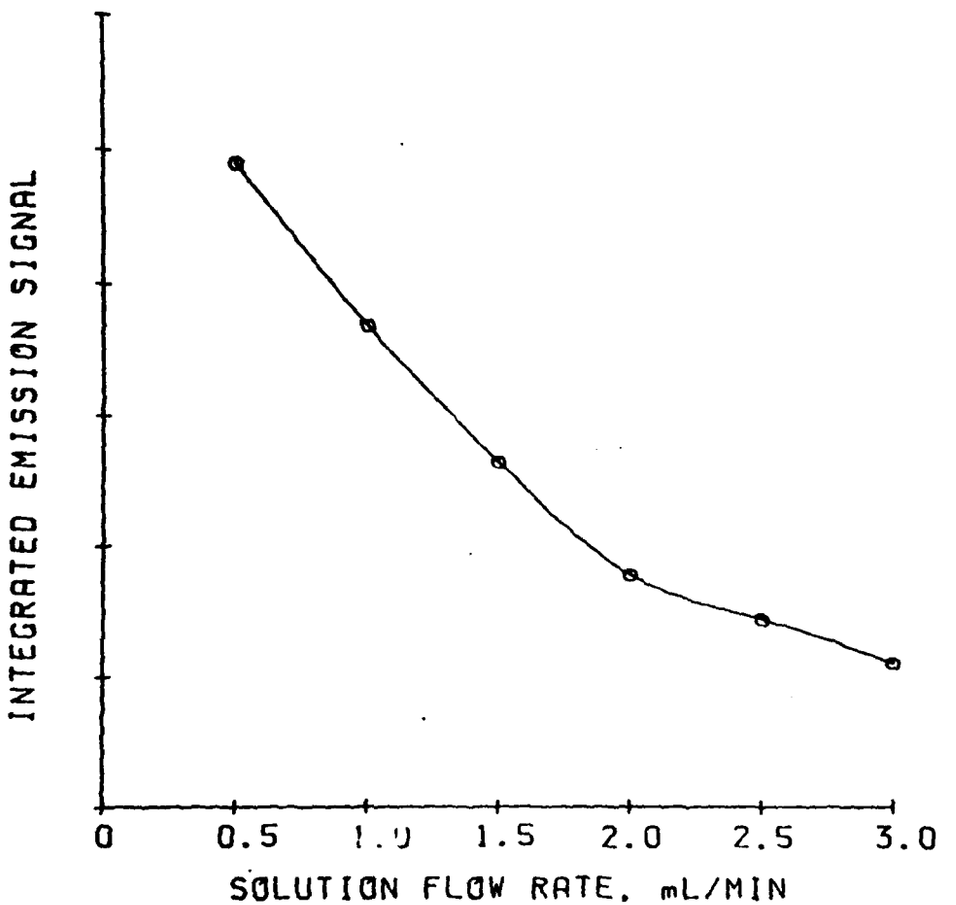
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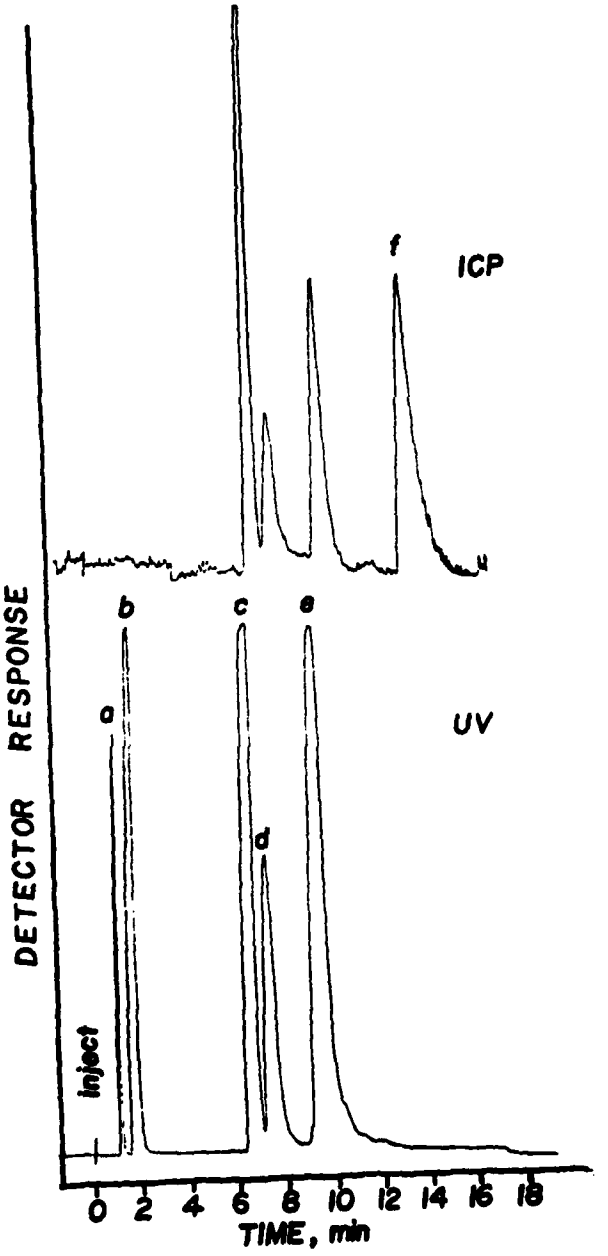
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